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Microalgae harvesting using ozoflotation: effect on lipid and FAME recoveries

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Abstract
Developing an economical algae harvesting system still remains a challenge today. One of the strategies to decrease harvesting costs prior conversion is employing technologies that can have additional benefits apart of separation. In this work we explore ozoflotation as a technique to recover microalgae grown in treated wastewater and we evaluate the effect of ozone on lipid and FAME recoveries. A high percentage of biomass can be harvested (79.6% as TSS) when using an ozone dose of 0.23 mg/mg of dried biomass. Additionally, two interesting effects of ozone were found in this study. The amount of lipid extracted and FAME recovered from the biomass, at least, doubles when using ozoflotation (with ozone doses of 0.12-0.23 mg/mg of dried biomass) than when using centrifugation. And, the oxidative stability of biodiesel can be enhanced by the effect that ozone has in the degree of FAME saturation.
Keywords: ozoflotation, microalgae, biodiesel, harvesting.

1. Introduction

In recent years, there has been increased research in the potential of using microalgae for biodiesel production. One of the limitations in the microalgae process is the operational costs needed before obtaining biodiesel. Reusing wastewater has been proposed as a way to decrease microalgae growth costs, as nutrient removal from wastewater and carbon sequestration can also be achieved. Pittman et al. [1] reviewed the potential of algal biofuel production and concluded that according to current technologies, algae cultivation for biofuels without the use of wastewater is unlikely to be econonomically viable.
After growth, biomass harvesting, is estimated to account between 20 to 30% of total biodiesel production costs [2]. The increased harvesting cost is a consequence of the low concentrations of microalgae in solution (1 to 8 g/L) obtained in cultivation systems [3], small size of microalgae cells (3 to 300 μm) and low microalgae density differences with culture water (average ~1020 g/L) [4], [5]. Developing an economical algae harvesting system remains a challenge today, the most common harvesting techniques include centrifugation, filtration, gravity sedimentation, dissolved air flotation, and flocculation; or a combination of processes [2], [6], [7].
One of the strategies to decrease harvesting costs prior conversion is employing technologies that can perform several treatments apart from separation. In this case, the harvesting of microalgae grown in wastewater could be combined with water treatment by

using technologies that perform both. Ozoflotation is a process currently utilised in wastewater treatment when separation needs to be combined with organic matter oxidation. In this ozoflotation process, bubbles of ozone, provided by diffusers, adhere to suspended solids (e.g.microalgae), break the superficial tension, and travel to the upper part of the bulk liquid to be physically separated. There have been previous reports using ozoflotation for microalgae separation in drinking water, to ensure that the toxic metabolites produced by microalgae are oxidised in water for drinking purposes [8], [9].

There is limited literature on the use of ozone for harvesting microalgae. However, the concept of combininig the oxidizing properties of ozone with flotation process has been applied by Van Vuuren et al. [10], and Bourbigot et al. [11]. Betzer et al. [12] reported removal of algae, TSS and total coliform from wastewater effluent by ozone-induced flotation. Ozone is a strong oxidizing agent that can oxidize double bonds and electron-rich aromatic rings to compounds like ketones, aldehydes and carboxylic acid [13], [8]. Ozone could have reacted with the organic matter in water via two pathways: direct ozone attack on the acid by molecular ozone and indirect ozone action through a variety of high-power oxidative radicals, which can act as secondary oxidants [14], [15]. In the first oxidation pathway, the direct ozone attack is followed by an electrophilic aromatic substitution and undergoes very selective reactions. The second oxidation pathway is less selective than the first pathway and facilitated by the action of radical species. Those radicals are generated from the ozone decomposition, as hydroxyl radicals, and leads to fast reactions with organic compounds.

Separation of microalgae using ozoflotation is advantageous over dissolved air flotation and gas bubble flotation because it does not require a flocculant or to lower the pH [12]. The effect of ozoflotation on microalgae lipid or fatty acid methyl esters yields has not been fully explored although it has been shown that the type of harvesting technology can affect the biofuel produced from microalgae [16], [17], [18]. In this work, we evaluate the use of ozoflotation as a separation process for microalgae recovery and its effect in lipid yields and ultimately microalgae FAME production and composition.

2. Methodology

2.1 Microalgae samples

Eight sampling visits were done during the months of Autumm (September, October, November) and Spring (February, March and April) to the Nabor Carrillo Lake located in Texcoco. In the visits, fresh treated wastewater containing native mixed microalgae were sampled from the lake and immediately used for experiments. The Lake is located in a high salinity soil and receives effluent from a wastewater treatment plant consisting of two facultative lagoons. Water characterisation was done according to Standard Methods for the examination of water and wastewater [19] in terms of: pH, temperature, conductivity, turbidity, ammonia, nitrates, orthophosfates and total suspended solids (TSS), Microalgae species were identified using a microscope (Leitzborlux S, Germany) and their concentration was determined using a Neubauer camera.

2.2 Evaluation of ozoflotation for microalgae harvesting

Ozoflotation experiments were conducted immediately after sampling, using 950 ml of fresh homogenised treated wastewater containing a native culture of microalgae. Batch flotation tests were performed in a glass column reactor (height: 66 cm; inner diameter: 4.9) designed for microalgae ozoflotation with a volume of 1.3 L. Ozone was generated in a

LABO 76, (Emery-Trailligaz Co, USA), using oxygen-enriched air as feeding gas (90% ± 5% purity) which was supplied by an Air Separator AS-12 (AirSep Co, USA).
Ozone was injected using a glass diffuser (10-15 μm pore-size) located at the bottom of the reactor. During the experiment the foam formed accumulated microalgae at the top of the reactor and was collected using a side exit connected to a collection vessel. Once the contact time finished and microalgae biomass were separated, the turbidity, microalgae concentration and total suspended solids of the remaining bulk liquid (wastewater) were measured again. A factorial experimental design 3^k was selected to evaluate the effect of experimental conditions over the microalgae separation efficiency. Each factor was studied using 3 levels; low, medium and high (-1, 0 and 1) respectively, levels were defined taking into account preliminary tests. Conditions studied were: ozone flowrate (0.2, 0.4 and 0.6 L/min), ozone gas concentration in the gas phase (25, 35 and 45 mgO_3/L) and ozoflotation time (5, 10, 15 min). Response factors were the Total Suspended Solids (TSS) and turbidity removals. Experiments were done in duplicate giving a total of 54 runs.
TSS and turbidity removal efficiencies obtained from the experimental design were analysed using an analysis of variance (ANOVA) to determine the signifance of tested variables in ozoflotation. A Fisher's Least Significant Difference (LSD) test at $P \leq 0.05$ was carried out to denote the statistical differences between the three factors. Statistical analyses were performed using the Minitab version 16 and Statgraphic Centurion XV.

2.3 Effect of ozoflotation in lipids extracted and Fatty Acid Methyl Esters (FAME) produced by microalgae

Ozoflotation effect on microalgae lipid extraction and FAME production were evaluated by changing the ozone concentration in the gas phase and fixing the ozone contact time to 5 min and flowrate to 0.4 L/min. In this case, a wide range of applied ozone doses were evaluated (0-140 mgO_3/L of waswater), as the highest applied ozone dose previously tested (94.7 mg O_3/L of waswater) produced the highest TSS removal in the previous experimental design. Ozoflotation was conducted as previously explained and microalgae was analysed as detailed in the following sections 2.3.1 and 2.3.2.

2.3.1 Drying of microalgae and lipids extraction

Algal samples collected by ozoflotation were centrifuged at 15,000g at 4 °C for 10 min using a Beckman Coulter centrifuge (mod. J2-21), then the pellet was frozen with dry ice at -70 ° C to be subjected to lyophilization (LABCONCO Liofilizadora Free Zone 4.5) under a temperature of -50 °C and vacuum pressure of 0.180 mba. Following this, lipids were extracted from microalgae and analysed. Lipid extraction was performed using a homogeneous solution of chloroform-methanol ratio of 2:1 (v/v) [20]. The volume of the chloroform and methanol solution was 45 times the mass of the solution. Therefore, for 1 g of algae biomass, the volume of chloroform and methanol was 30 ml and 15 ml, respectively. The solution was left overnight in the fridge at a temperature of 4 ^oC. After the overnight extraction, the samples were vacuum-filtered using a Whatman glass microfiber filter paper of 70 mm diameter. The filtrates were poured into separating funnels and a weak salt solution of potassium chloride (KCl; 0.88 vol%) was added at 25% of the starting weight. The solutions were well mixed and allowed to separate into two layers. The lipid layers were carefully removed into pre-weighed conical flasks and left to dry in a fume

cabinet until constant weight. The mass of lipid in microalgae species was finally obtained by deducting the vessel weight from the final constant weight.

2.3.2 Conversion of microalgae lipids to FAME and analysis.

Lipids contained in dried microalgae samples were converted to FAME by the one-step FAME extraction as used previously by our group [21]. The method consisted on preparing a methylating mixture by mixing methanol: toluene: 2, 2-Dimethoxypropane: sulphuric acid (39:20:5:2 by volume). The methylating mixture (3.3 ml) was then mixed with heptane (1.7 ml) and added to 0.2 g of dried biomas of microalgae followed by vigorous shaking (450 rpm) and incubation at 60°C for 12 h. After this, the sample was cooled down and the upper layer formed in the weighted mixture.

Gas chromatography was used to quantify and profile the FAME produced. The gas chromatograph used was the Hewlett Packard 5890 series II fitted with a FID detector. The run time selected was 30 minutes using helium as a carrier gas. Air and hydrogen input pressures were set at 48.26kPa, 220.63kPa and 151.68 kPa, respectively and column head pressure was fixed at 31.03 kPa. A sample of the upper layer (250 mg) was mixed with 250 µl of the standard solution (C17:0 sigma Aldrich, 10 mg/ml) in a 2 ml vial. Before injecting 5 µl of the sample mixture, 5 µl of methanol and 5 µl of standard solution (C17:0) were sequentially injected to ensure a clean column, and to check C17 retention time and purity. All data was collected using DataApex Clarity software. Once all samples were injected, 3 µl of the grain FAME mix (Sigma Aldrich, 10 mg/ml) were analysed to enable classification of the peaks obtained. FAME concentrations were obtained as a mass fraction in percent of the total sample weight (including methanol), using the following equation:

$$C = \frac{(\sum A) - A_{EI}}{A_{EI}} \times \frac{C_{EI} \times V_{EI}}{m} \times 100\%$$

Where:

$\sum A$: Is the total peak area
A_{EI}: Is the peak area corresponding to methyl heptadecanoate (C17:0)
C_{EI}: Is the concentration, of the methyl heptadecanoate solution (mg/ml)
V_{EI}: Is the volume, of methyl heptadecanoate solution (ml)
m: Is the mass, of the sample (mg)

3. Results and discussion

3.1 Microalgae present in artificial Nabor Carrillo Lake

It has been demonstrated that microalgae can successfully grow in wastewater and produce biomass with high lipid content,suitable for biofuel production, with the added benefit of wastewater treatment [22], [23]. In this case, the lake water was refilled using treated wastewater and had various characteristics that promote microalgal growth, such as: alkaline pH, high orthophosphate and nitrogen (Table 1). Phosphorus can be taken in the forms of $H_2PO_4^-$ and HPO_4^{-2} during algae metabolism and incorporated as structural organic compounds [24]. Microalgae developed in the lake despite the high salinity found in both seasons. This is in agreement with Osundeko et al. [25] who demonstrated that naturally adapted microalgae strains have an increased tolerance to stressed conditions and can have an efficient grow in treated wastewater despite some external environmental changes.

Figure 1 shows the major genera of microalgae identified in artificial lake Nabor Carillo. Most of the genus of mixed microalgae were present in the lake in both seasons, except for *Merismopedia* and *Pediastrum* that were only present during the Autumn. The similar types of cultures present can be explained by the comparable charateristics found in wastewater effluents (Table 1). Microalgae species of *Oscillatoria* and *Cyclotella* were the most abundant in both seasons. In terms of quantity, microalgae were more abundant during the Spring season. *Oscillatoria* are genera in the phylum cyanobacteria, and they are fast growing. *Cyclotella* belong to the phylum of bacillariophyta has been identify to grow under increased salinity conditions up to 45% [26].

3.2 Microalgae harvested using ozoflotation

The effect of ozone on the cells was observed after two minutes of gas contact time, when the biomass began to produce a foam in the top of the reactor and a concentrate started to be released into the collection vessel. A similar effect was reported by Cheng et al. [16] when applying ozone. The formed upper froth layer with floating algae was attributed to the release of microalgae proteins. These proteins can act as surfactant by increasing the hydrophilicity of bubble surfaces for the effective bubble-cell collision.

Table 2 depicts the experimental signifance of ozoflotation tested variables (ozone flow rate, ozonation time and ozone concentration in the gas phase) on TSS and turbidity responses. Regarding TSS, 6 effects have p-values less than 0.05 indicating that they are significant at a 95% confidence level. Within the 6 effects, ozone concentration in the gas phase and the ozoflotation time have a p-value of 0.00 indicating that they were the main variables affecting TSS removal. Regarding turbidity, 3 effects have p-values lower than 0.05; and only ozone concentration in the gas phase has a p-value of 0.00. These results indicate that within the tested variables the degree of significance for the tested factors has the following order: ozone concentration in the gas phase > ozonation time > ozone flow rate, and therefore ozone concentration in the gas phase is the main variable affecting ozoflotation in the range of conditions tested.

Figure 2 A shows that using an ozonation time of 5 min produces the same degree of TSS removal as an ozonation time of 15 min at an ozone gas concentration of 45 mg/L. Equally, at an ozone gas concentration of 45 mg/L, a flow of 0.4 mL/min produced the same degree of TSS removal as a flow of 0.6 mL/min. It is therefore concluded that a flow of 0.4 mL/min and an ozonation time of 5 min should be enough to produce the highest removals at an ozone gas concentration of 45 mg/L. This best condition value can also be seen in

Figure 2 B which shows that the highest amount of TSS removal (79.6%) was obtained with an ozone gas concentration of 45 mg/L, flowrate of 0.4 L/min and 5 min of ozonation (equivalent to an ozone dose of 94.7 mg/L of waswater and 0.23 mgO_3/mg of dried biomass). The harvested biomass by ozone was contained in a concentrated volume of 20 ml, achieving a concentration factor of, approximately, 48 times.

The results of this study compared with those published by other authors are shown in Table 3. It can be seen that the optimal ozone dose obtained in the harvesting of a mixed culture of microalgae from Nabor Carrillo Lake was between two to four times higher than the maximum doses employed by Betzer et al. [12] and Cheng et al. [16], but in the range of doses reported by Cheng et al. [17]. The type of microalgae species used affect the required ozone dose. Cheng et al. [16] and [17], report a higher ozone dose to harvest *Scenedesmus obliquss* FSP-3 than that for *Chlorella vulgaris* (Table 2). It is proposed that the difference in the ozone doses required are due to the different humic substances found in the suspension as they can scavenge the dosed ozone and adversely affect the ozoflotation of algal cells [17].

3.4 Ozone effect on microalgae lipids

The purpose of harvesting algae by ozoflotation is to obtain biodiesel precursor lipids in the algal biomass, therefore the effect of ozone on total lipid content was determined. When the microalgae biomass was harvested by centrifugation, the amount of extracted lipids was 2.35% and 5.85%, in Autumn and Spring, respectively.

The amount of extracted lipids, increased with the ozoflotation according to the applied ozone dose (Figure 3), but this increase was not linear and presented an optimum value. A total maximum lipid of 12% was obtained when using an applied ozone dose range 52.6-94.7 mg O_3/L (equivalent to 0.12-0.23 mgO_3/mg of dried biomass dry biomass), however ozone doses greater than 100 mgO_3/L (> 0.24 mgO_3/mg of dried biomass), decreased the amount of lipids recovery. Considering, both, the harvested SST and lipids recovery, a dose of 0.23 mgO_3/mg dry biomass will be recommended to achieve the maximum recovery. It is possible that higher doses of ozone caused increased cell lysis thereby releasing lipids into the supernatant. This will be investigated in further work. The results obtained in the biomass harvested with ozone, suggest that the ozoflotation of microalgae, improves lipid extractability. It has been shown that ozone can lyse cells of microalgae [8], which may contribute in lipid extraction.

3.5 Effect of ozoflotation on FAME composition

The types of FAME obtained by transesterification of lipids from microalgal biomass for the two seasons (Autumn and Spring) are shown in Table 4. The highest percentage of total lipids, per dry weight biomass of mixed cultures, was obtained in Spring samples. Palmitic acid (C16:0) was the most abundant FAME followed of linolenic, linoleic, palmitoleic and lauric fatty acid methyl esters.

Table 4 also shows the effect of ozoflotation harvesting on FAMEs composition after microalgae transesterification. When ozone was used as a method of harvesting, more compounds were identified in the fatty acid profile than when using centrifugation. A first ozone effect was reflected in the type of FAMEs obtained. There was a marked increase on palmitic acid mass and concentration (C16:0) when using ozoflotation than when using centrifugation (also seen in Figure 4). As it was the case for lipid production, the FAME yield increased with the ozone dose until reaching a peak at 63.2 mgO_3/L and then started

to decline at 94.7 mgO_3/L. The best ozoflotation condition for FAME production at 63.2 mgO_3/L of wastewater, primarily produced C16:0 (1.85%w), C18:1n9c (0.39%w), C16:1n9c (0.34%w), and C18:3n3 (0.31%w). However, considering TSS, lipid and FAME yields, the recommended condition remained at 94.7 mgO_3/L (equivalent to a dose of 0.23 mgO_3/mg dry biomass). A second ozone effect was shown in the degree of FAMEs saturation. The percentage of saturation, in the total mixture of FAMEs, increased significantly from 52.5% (microalgae harvested by centrifugation) to 81.1% in ozoflotated microalgae with 142.1 mgO_3/L of wastewater. The increased saturation is mainly due to decreasing levels of polyunsaturated acids such as linolenic acid (C18:3n3), and linoleic acid (C18:2n6c). An increased FAME saturation is desirable as it elevates the oxidative stability of the biodiesel produced from microalgae [27]. In fact, according to the Standard EN 14214 linolenic acid (C18:3n3) percentage must be under 12%, a condition that is only met for this feedstock when using ozoflotation (Figure 4). The evolution of the main FAME compounds as the ozone dose increases (C16:1n9c, C18:1n9c, C18:2n6c and C18:3n3) indicated that unsaturated lipids, with two or more double bonds in their structure, favour the reaction with ozone. It is known that ozone oxidizes double-bonded compounds into oxygenated functional groups like ketones and aldehydes [8]; therefore, a decrease in the percentage of unsaturated FAME was expected. An increased number of oxygenated groups in biodiesel FAMEs may also affect the quality of biodiesel by reducing its energy density but improving its burning characteristics. It is concluded that the FAMEs obtained from the microalgal biomass harvested by ozoflotation should require less refining than when harvested by centrifugation.

Conclusions

Nabor Carrillo Lake fed with secondary effluent presented a variety of microalgae cultures. *Oscilatoria* strains were predominant in Spring, and *Oscilatoria and Cyclotella* strains prevailed during the Autumn. The maximum harvesting percentage achieved was 79.6% (as TSS removal), at an ozone dose of 0.23 mgO_3/mg biomass. An ozone dose of 0.12 mgO_3/mg of dried biomass increased the amount of extracted microalgae lipids from 6% (when using centrifugation) to 12% and the FAME yield from 1.23% (when using centrifugation) to 3.23% w of FAME/ by w of dried microalgae. The recovery of mixed microalgae by the ozoflotation process also had a positive effect on the conversion of lipids to FAME. In general, FAME concentrations increased when using ozone compared to using centrifugation. Considering the harvested SST, lipids recovery and conversion to FAME a dose of 0.23 mgO_3/mg dry biomass, will be recommended. Ozoflotation is presented as a multifunctional downstream processing technique in the conversion of microalgae to biofuels: it can not only separate water from microalgae biomass, but also increase the efficiency of lipid extraction from microalgae biomass, and may also improve the FAME composition produced from mixed microalgae.

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Figure 1

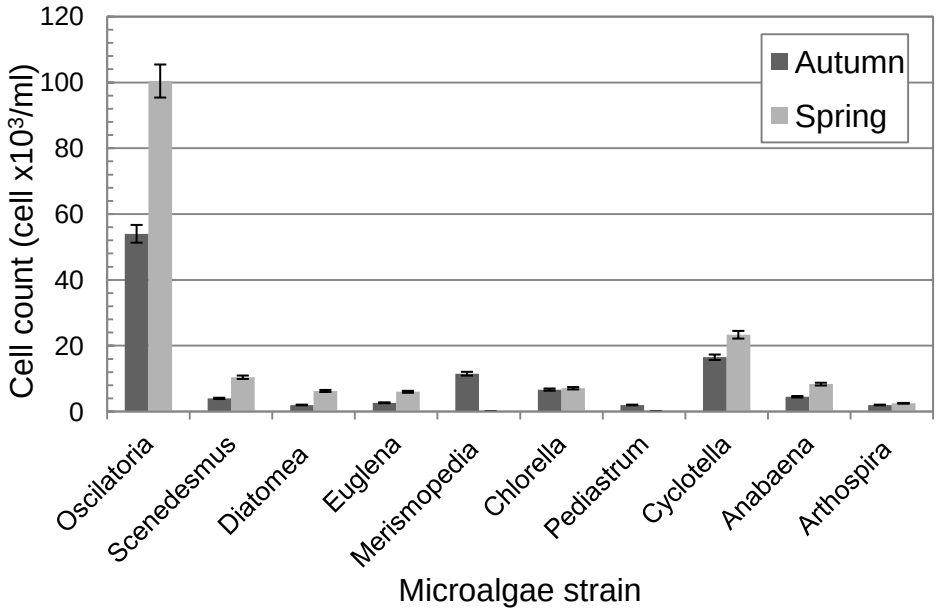
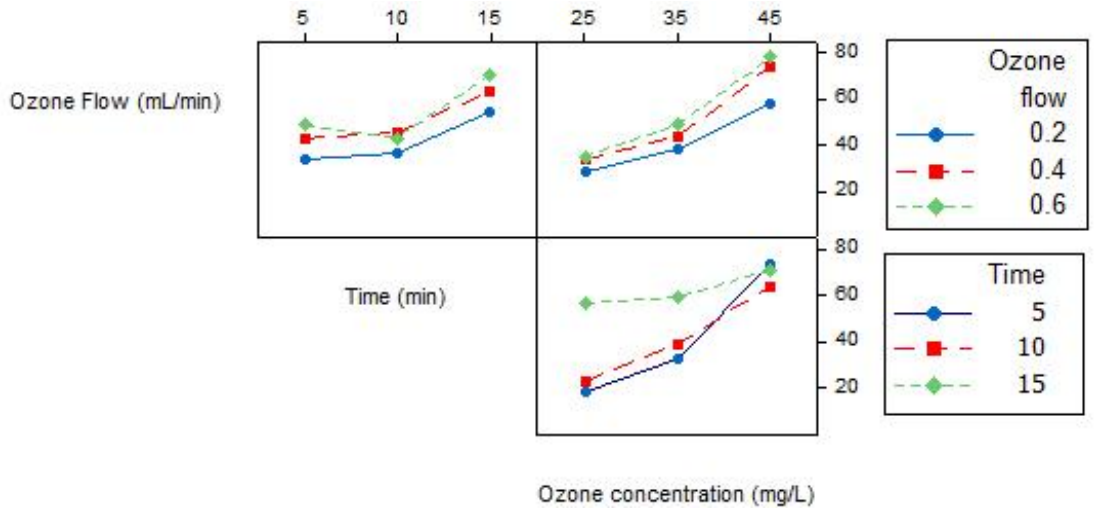

120
100
80
60
40
20
0
Cell count (cell x10³/ml)
Autumn
Spring
Oscilatoria
Scenedesmus
Diatomea
Euglena
Merismopedia
Chlorella
Pediastrum
Cyclotella
Anabaena
Arthospira
Microalgae strain

Figure 2

A)


5
10
15
25
35
45
Ozone Flow (mL/min)
Time (min)
80
60
40
20
Ozone
flow
0.2
0.4
0.6
Time
5
10
15
Ozone concentration (mg/L)

B)

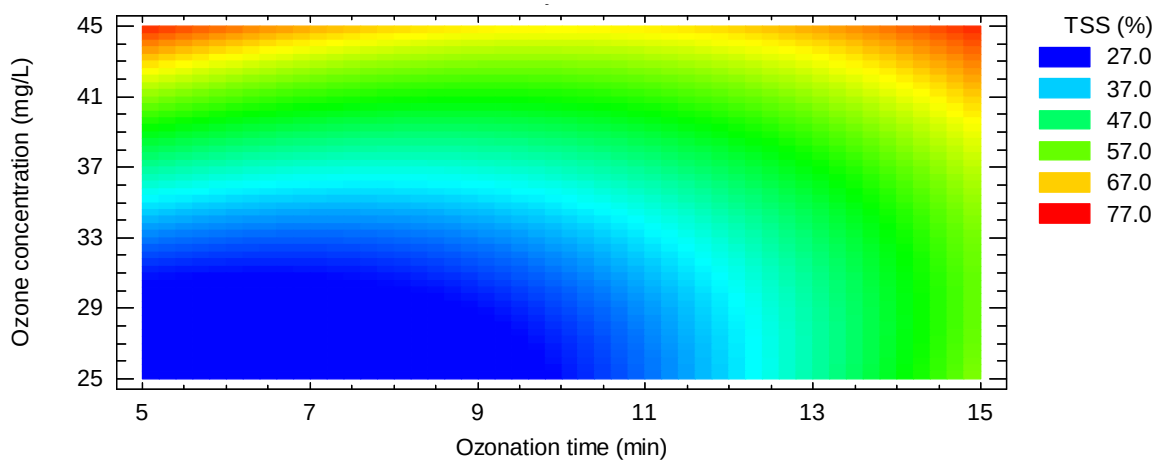

45
41
37
33
29
25
Ozone concentration (mg/L)
5
7
9
11
13
15
Ozonation time (min)
TSS (%)
27.0
37.0
47.0
57.0
67.0
77.0

Figure 3

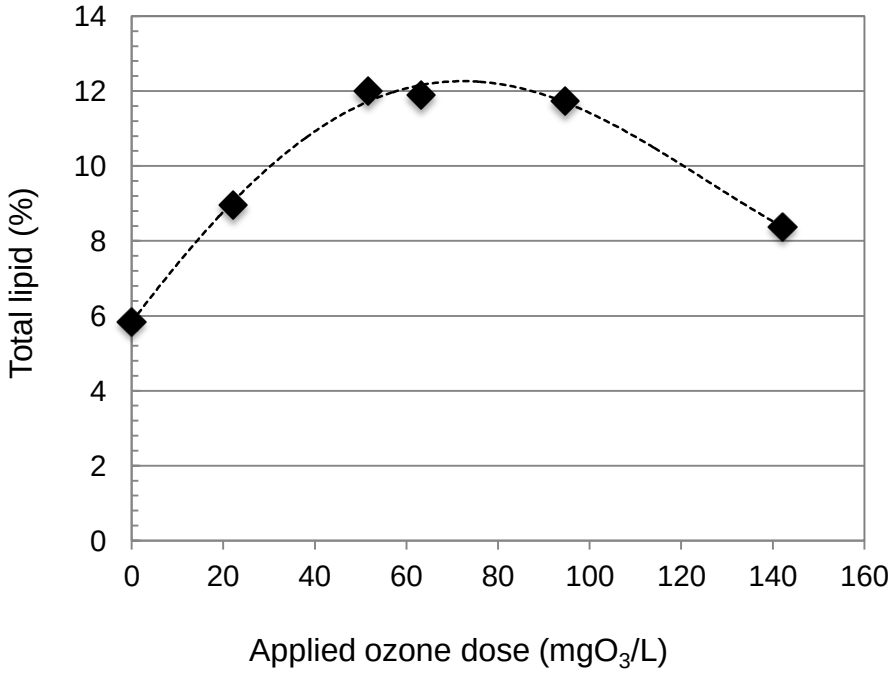


Figure 4

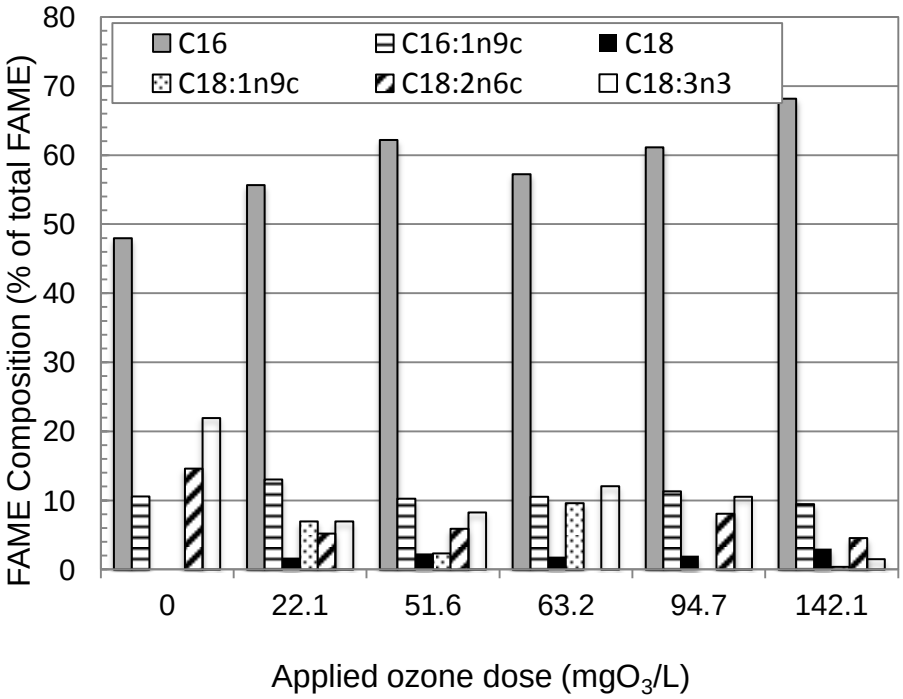


Table 1 Water Quality of the Nabor Carrillo lake.

Parameter	Autumm	Spring
pH	8.55 ± 0.62	9.54 ± 0.42
T(^{o}C)	18.65 ± 3.04	21.0 ± 0.99
Turbidity (NTU)	447.50 ± 18.70	712.50± 46.59
Conductivity(µS/cm)	3100 ± 450	4840 ± 110
Ammonia (mg/L)	1.32 ± 0.17	2.14 ± 0.15
Nitrates (mg/l)	0.55 ± 0.049	0.67 ± 0.052
Orthosphates(mg/l)	8.40 ± 0.537	10.12 ± 0.75
Total suspended solids (mg/l)	388.5 ± 19.70	419.0 ± 23.0

Table 2 Experimental significance of tested variables in ozoflotation

	TSS		Turbidity	
Source	F-Ratio	P-Value	F-Ratio	P-Value
A: Flow	10.98	0.0041	19.55	0.0004
B: Time of contact	29.81	0	13.27	0.002
C: Ozone concentration	96.88	0	95.43	0
AA	0.68	0.4197	2.23	0.1534
AB	0.03	0.8636	3.99	0.0621
AC	2.21	0.1551	5.38	0.0331
BB	9.6	0.0065	5.98	0.0257
BC	19.91	0.0003	3.43	0.0814
CC	5.07	0.0379	6.41	0.0215

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